



# Milk phospholipid antioxidant activity and digestibility: Kinetics of fatty acids and choline release



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## ABSTRACT

Milk phospholipids have been used as functional ingredients in foods. However, there have been no reports on how the polarity of lipids affects their digestibility. This study aims to isolate milk phospholipids from dairy products, to characterize them chromatographically, and assess their digestibility and antioxidant activities *in vitro*. The results revealed that their lipolysis reaction rate constants were significantly different ( $p < 0.05$ ) between milk phospholipids and triacylglycerols, and thus the degradation of both lipids complied with first-order reaction kinetics. Furthermore, the cellular uptake of milk phospholipids was determined with HT-29 cell model, and they were not found to be absorbed intact during intestinal digestion. Milk phospholipids exhibited significant antioxidant activity *in vitro*, while their cellular antioxidant activity was very limited. The results of this study provide useful information for the design of milk phospholipid-based carrier systems.

## 1. Introduction

Phospholipids are important components in milk and they construct the backbone of the tri-layer milk fat globule membrane. They serve as functional nutrients with both technical importance and nutritional relevance (Oliveira, Puri, Fenelon, & O'Mahony, 2019; Zheng, Jiménez-Flores, & Everett, 2014). For instance, milk phospholipids have been used as vesicle materials since the early 2000s. Thompson, Couchoud, and Singh (2009) used milk phospholipids to fabricate three kinds of liposomes to carry bioactive molecules. Since then, milk phospholipid-based liposomes have been fabricated to deliver ascorbic acid (Farhang, Kakuda, & Corredig, 2012) and lactoferrin (Liu, Ye, Liu, Liu, & Singh, 2013). More recently, milk phospholipid liposomes have been applied to improve the storage stability of encapsulates (Gulseren & Corredig, 2013), the encapsulate solubility and encapsulation efficiency (Jin, Lu, & Jiang, 2016; Liu, Ye, Liu, Liu, & Singh, 2012), and the bioavailability of encapsulate (Maswadeh, Aljarbou, Alorainy, Alsharidah, & Khan, 2015), with milk phospholipids showing better efficiency compared to soy lecithin (Liu et al., 2012). Phospholipids have been used to prepare antioxidant phytosomes (Mancini et al., 2018) and to modulate the release of antioxidant from phospholipid vesicles (Vu et al., 2018). The

digestion kinetics of phospholipids govern the bioavailability of encapsulated bioactive compounds and have an impact on their release (Zhang, Zhang, Zhang, Decker, & McClements, 2015). Milk phospholipids have unique composition and characteristics, which may affect the release of bioactive compounds along the gastrointestinal tract (Arranz & Corredig, 2017).

Aside from the vesicle properties, milk phospholipids have been refined as functional ingredients. Phospholac 500/600/700 and Gangolac 600 have purities of 33.9%, 69.8%, 61.6%, and 14.5%, respectively (Li, 2014; Thompson, 2005), while Lacprodan® MFGM 10 and Lacprodan® PL 20 (purity 20%) have been produced for functional food ingredients (Burling & Graverholt, 2008) and infant formulas (Sokol, Ulven, Faergeman, & Ejsing, 2015). Lipidex, a derivative from beta serum powder, contains 5 – 7% phospholipids and 26.6% fat in total (Moukarzel, 2016), and bovine milk sphingomyelin has a purity of 99% (Lopez, Briard-Bion, & Ménard, 2014). In addition, Lipamine M20, which has been manufactured with membrane separation, comprising 20% of sphingomyelin, ceramides, and ganglioside (Lecico, 2019). Ethanol is the most used solvent to extract milk phospholipids, for instance, hot alcohol (70%) extraction at 70 °C rendered 45.8% purity, starting from whey protein phospholipid concentrate (Price, Fei, Clark,

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& Wang, 2018). A switchable hydrophilicity solvent, tertiary amine, was used to extract phospholipids from buttermilk, resulting in 99% extraction efficiency (Cheng, Rathnakumar, & Martinez-Monteagudo, 2019). In a laboratory up-scaling test on buttermilk powder substrate, a pilot unit integrated enzymatic proteolysis, ultrafiltration, and supercritical fluid extraction, yielding a dry-matter purity of  $56.24 \pm 0.07\%$  (Barry, Dinan, & Kelly, 2017; Barry, Dinan, & Kelly, 2017).

There have been a few reports on the digestibility of highly-purified milk phospholipids. For example, phospholipase A<sub>2</sub> is the enzyme responsible for phospholipid hydrolysis, but milk phospholipid liposomes disintegrate slower than soy lecithin liposomes (Arranz & Corredig, 2017; Liu et al., 2012). There have also been no reports on how polarity impacts lipid digestion thus far. Therefore, the present study aims to extract and refine phospholipids from dairy products, characterize them chromatographically, evaluate *in vitro* lipid digestibility, measure the cellular uptake in HT-29 cells, and assess *in vitro* antioxidant activity.

## 2. Materials and methods

### 2.1. Materials

Milk phospholipid concentrate (20.81% and 51.3% (w/w) lipids and proteins, respectively) was donated by Tatua Co-Operative Dairy Company (Morrinsville, New Zealand). Lipase L3126, porcine pancreas type II (EC232-619-9, CAS 9001-62-1, protein,  $\geq 20\%$ ), bovine bile (B3883, dried, unfractionated, 70% bile salts, 22% phospholipids, 4% cholesterol, 3% proteins and 0.3% bilirubin), and phospholipid assay kits (MAK122), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), dichlorodihydrofluorescein diacetate (DCFH-DA), thiobarbituric acid (TBA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich China. Lipopan F (25–50 kLU/g, active at pH 5–7) was supplied by Novozymes Company. A triacylglycerol assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing China. Finally, HT-29 cells (human colorectal adenocarcinoma cells with epithelial morphology, sourced from ATCC) were seeded in Tianjin Key Laboratory of Food and Biotechnology, Tianjin, China. The other chemicals used in this study were of analytical grade.

### 2.2. Extraction and purification of milk phospholipids

#### 2.2.1. Milk phospholipids extraction

Milk phospholipids were extracted using a modified Folch method (Nollet & Toldrá, 2015). Milk powder (20 g) were first dissolved in 450 mL methanolic chloroform (1:2, v/v). After 20 min of agitation, 160 mL saline aqueous solution (0.74 g NaCl in 100 mL solution) was added to precipitate the casein proteins. Following another 20 min of mixing, the mixture was centrifuged at 4000g for 30 min at 4 °C. Subsequently, the aqueous supernatant was withdrawn with a 5 mL pipette, while the solidified layer was removed with a scraper. Milk lipids in the chloroform layer (denser bottom layer) were dried under vacuum. The lipids were thrice defatted with acetone (Nwachukwu & Aluko, 2019). The acetone-insoluble precipitate was lyophilized to obtain purified phospholipids.

#### 2.2.2. Solid phase extraction

The milk phospholipid fractionate was further purified from neutral lipids by solid phase extraction (SPE) (Contarini, Pelizzola, Scurati, & Povolova, 2017). The lipid solution (100 mg phospholipids in 1 mL of *n*-hexane) was first loaded on a silica gel SPE cartridge (500 mg, 6 mL). After the column was washed with *n*-hexane, the non-polar lipids were eluted with 10 mL of *n*-hexane-diethyl ether (1:1, v/v). Next, the phospholipids were recovered by two steps of elution, firstly with methanol (10 mL) and followed by a solvent mixture of methanolic chloroform and water (5:3:2, v/v/v, 10 mL). The eluted phospholipids were finally dried under a gentle flow of nitrogen.

### 2.3. Chemical analysis

#### 2.3.1. Chemical composition analysis

Total lipid content of milk phospholipids was gravimetrically analysed, and while the contents of proteins, triacylglycerols, and phospholipids of each fraction were determined by the Bradford assay (Nwachukwu & Aluko, 2019), the triacylglycerol enzymatic colorimetric kit (glycerine phosphate oxidase peroxidase (GPO-PAP) (Norris et al., 2003)), and the phospholipid assay kit MAK122 (Shrestha et al., 2017), respectively.

#### 2.3.2. Thin layer chromatography

Milk phospholipids in chloroform solution (2  $\mu$ L, 100 mg/mL) were pipetted to the bottom edge of a silica gel thin layer chromatography (TLC) plate. The subclasses of milk phospholipids were separated with a solvent mixture (chloroform, methanol, and water, 65:25:4, v/v/v). Each band of polar lipids was oxidized in iodine vapour to visualize as coloured spots (Astaire, Ward, German, & Jiménez-Flores, 2003).

### 2.4. In vitro digestion

#### 2.4.1. Simulated intestinal digestion catalysed by pancreatic lipase and fungal lipase

Milk phospholipids were dispersed using heating method (Thompson, Mozafari, & Singh, 2007), with minor supplementation—sonication. The milk phospholipids aqueous dispersion was agitated at 160 rpm for 60 min at 80 °C. Subsequently, the phospholipid emulsion was further treated with sonication for 10 min to gain fine droplets (Sonics & Materials, Inc., 240 W, 20 kHz) (Vélez, Perotti, Zanel, Hynes, & Gennaro, 2017). The samples were analysed immediately following preparation. Milk lipid dispersion was prepared using the same heating method as well. Milk lipid dispersion (1 g milk phospholipids or 1 g milk triacylglycerols in 10 mL aqueous dispersion) was diluted with 30 mL of distilled water. The simulated intestinal fluid (SIF) was composed of dipotassium phosphate (0.68 g per 100 mL SIF), 150 mM sodium chloride and 30 mM calcium chloride (Liu et al., 2013), and SIF was adjusted to pH 7.0 using 0.1 M NaOH. Lipase (40 mg porcine pancreatic lipase or fungal lipase Lipopan F) and bile extract (200 mg, present for pancreatic lipase-catalysed samples, while not present for Lipopan F lipase-catalysed samples) were added to each pot to initiate the hydrolysis reaction. Aliquots of the digestion were taken at 0, 20, 40, 60, 90 and 120 min for composition analysis. The free fatty acids (FFA) were extracted with ethanolic diethyl ether, and then quantified titrimetrically with 0.1 M ethanolic potassium hydroxide (Hur, Lim, Park, & Joo, 2009).

Dietary phospholipids remain intact in the presence of lingual and gastric lipases (Castro-Gómez, García-Serrano, Visioli, & Fontecha, 2015). Gastric pH and salts can alter both the size and the structure of particles, which further affect the intestinal digestion. However, gastric digestion contributes to only a minor proportion (1.5–8%) of the total phospholipid hydrolysis (van Hoogevest, 2017) and takes place at pH 1.5–3, which is incompatible with the present titration quantification since both gastric acid and free fatty acids consume titrant. Therefore, only intestinal digestion was surveyed this study, as also commonly performed by recent reports on lipid digestion.

#### 2.4.2. Release kinetics of free fatty acids from milk phospholipids

The experimental data was fitted using a linear regression model ( $-\ln(1 - x_{\text{lipids}})$  vs  $t$  (min),  $k$  as the slope of trend-line) with the initial concentration of fatty acids being zero. The reaction rate constant  $k$  ( $\text{min}^{-1}$ ) was described by Eq. (1):

$$kt = -\ln(1 - x_{\text{lipids}}) \quad (1)$$

where  $x_{\text{lipids}}$  was the conversion rate of the substrates to free fatty acids (FFA) (Butterworth, Warren, Grassby, Patel, & Ellis, 2012).

#### 2.4.3. Choline release from milk phospholipids

The phosphatidyl groups in milk phospholipids were cleaved by phospholipases D and A<sub>2</sub>, and pancreatic lipases in 96-well, flat-bottom microplates. The free choline was quantified at 570 nm using the colorimetric assay kit MAK122. The reaction rate constant was obtained using the same model used for fatty acid release, as described in Section 2.4.2 above.

### 2.5. Absorption and cytotoxicity

#### 2.5.1. Cell culture

The HT-29 cells (sourced from ATCC, stored at Tianjin Key Laboratory of Food and Biotechnology, China) were grown in Dulbecco's modified Eagle's medium (DMEM), which comprised of 10% (v/v) fetal bovine serum (FBS) and 0.5% (v/v) penicillin and streptomycin (both 100 U mL<sup>-1</sup>). The cells were first incubated at 37 °C and 5% CO<sub>2</sub> until their confluence. The confluent cells were then washed twice with phosphate buffered saline (PBS) after a removal of culture medium. Ethylenediaminetetraacetic acid (EDTA) solution (1 mL, 1%, w/v) was used to detach the cells from the well surface (1 min, 37 °C), followed by the addition of 1 mL of DMEM to neutralize the EDTA. The detached cells were then suspended with a pipette and transferred to a tube for centrifugation at 240g for 3 min at 4 °C. The supernatant was discarded, and the pelleted cells were again dispersed with 1 mL of culture medium. The density of the cells was determined with a cell counting plate. DMEM medium was replaced every three days.

#### 2.5.2. Cellular uptake of phospholipids

The method of determining the cellular uptake of ascorbic acid was adapted from a previous report (Yu et al., 2016). The confluent HT-29 cells (passage of 15 – 35) were seeded in culture dishes (50 mm in diameter) at a density of 0.2 million cells per petri dish. After 24 h of treatment of milk phospholipid dispersion (5 mL per petri dish, 1 mg/mL phospholipids), ice-cold PBS was used to wash the HT-29 cell monolayer twice, and then trypsin-EDTA solution (1 mL, 1%, w/v) was added to suspend the cells. Following an incubation (1 min), the EDTA was neutralized by addition of the growth medium (1 mL). The suspension was centrifuged at 240 g for 3 min to pellet the HT-29 cells. The supernatant was aliquoted to determine the milk phospholipid concentration (C<sub>f</sub>), and the cellular uptake (CU) of milk phospholipids was then calculated by Eq. (2):

$$CU = (C_i - C_f)/C_i \quad (2)$$

where C<sub>i</sub> and C<sub>f</sub> are the initial and final concentration of milk phospholipids, respectively.

#### 2.5.3. Anti-proliferation activity

Milk phospholipid toxicity was analysed colorimetrically using the MTT assay (Yu et al., 2016). HT-29 cells were seeded at a density of 5,000 cells per well in 96-well, flat-bottom microplates. The cells were incubated for 24 h (37 °C and 5% CO<sub>2</sub>) to be confluent. The cells were then treated for 3 h in a dispersion of milk phospholipids (2 µL per well, 0.025–0.1 mg/mL) supplemented with 20 µM MTT. Following incubation, the dispersion was gently removed with a 200 µL pipette, and the cells were carefully washed twice with cold PBS. Dimethyl sulfoxide (DMSO, 100 µL) was then added to each well to dissolve the formazan crystals inside the cells. The absorbance of each well at 490 nm was measured in a microplate reader. The relative cell viability (CV) was obtained by normalizing the absorbance of the treated cells (A<sub>t</sub>) with that of the control samples (A<sub>c</sub>), using Eq. (3):

$$CV = (A_c - A_t)/A_c \quad (3)$$

### 2.6. Antioxidant activity

#### 2.6.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was measured as previously described (Yilmaz-Ersan, Ozcan, Akpinar-Bayazit, & Sahin, 2018). Samples (250 µL) and 180 µL freshly-prepared DPPH (1 mM methanolic stock DPPH solution, 39.4 mg DPPH in 100 mL methanol) were added to methanol (2570 µL), and were then incubated in dark for 30 min to measure the absorbance at 515 nm (DPPH) with a spectrophotometer. The DPPH radical scavenging rate (SR) was calculated against reagent blanks, as expressed in Eq. (4):

$$SR(\%) = (1 - (A_t/A_c)) \times 100 \quad (4)$$

where A<sub>t</sub> and A<sub>c</sub> were the absorbance of the samples and controls at 30 min, respectively (Vital et al., 2018). Ascorbic acid was selected as the reference antioxidant.

#### 2.6.2. ABTS radical scavenging activity

A colorimetric assay was used to quantify the ABTS radical scavenging activity of milk phospholipids (Verma, Chatli, Mehta, & Kumar, 2018), in comparison to ascorbic acid. The mixture (1:1, v/v) of ABTS solution (7 mM) and potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 2.45 mM) was kept for 16 h in the dark to form the radical cation (ABTS<sup>•+</sup>) at ambient temperature. Subsequently, the optical density of the solution was adjusted to 0.70 at 734 nm with ethanol. The mixture of the ABTS working solution (1 mL, 0.7 in absorbance) and samples (100 µL) was then incubated for 20 min before its absorbance (A<sub>20</sub>) was measured at 734 nm. The ABTS radical scavenging rate (SR) was calculated using Eq. (5), where A<sub>blank</sub> is the absorbance of the reagent blanks:

$$SR(\%) = ((A_{blank} - A_{20})/A_{blank}) \times 100 \quad (5)$$

#### 2.6.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the milk phospholipids was measured with a spectrophotometric protocol as described by Zhu et al. (2019). PBS (1 mL, 0.4 M, pH 7.4), 1,10-phenanthroline (1 mL, 2.5 mM, Sigma-Aldrich), FeSO<sub>4</sub> (1 mL, 2.5 mM), and H<sub>2</sub>O<sub>2</sub> (0.5 mL, 0.01%, w/v) were added to the milk phospholipid dispersion (1 mL of various concentration). After 1 h of incubation at 37 °C, the absorbance of the samples was measured at 536 nm (1,10-phenanthroline-Fe<sup>2+</sup> complex). The scavenging rate (SR) of hydroxyl radicals was calculated as follows:  $SR(\%) = [(A - A_b)/(A_c - A_b)] \times 100$ , where A<sub>c</sub> was the absorbance of the controls (the samples and H<sub>2</sub>O<sub>2</sub> were replaced with 1.5 mL of distilled water), A<sub>b</sub> was the absorbance of the reagent blanks (the samples were replaced with 1 mL of distilled water), and A was the absorbance of milk phospholipid samples.

#### 2.6.4. Thiobarbituric acid reactive substance (TBARS) formation inhibition method

TBARS assay on milk phospholipids was determined according to the description by Murdifi et al. (2017), in comparison with both ascorbic acid (0.1 mg/mL) and butylated hydroxytoluene (BHT, 0.1 mg/mL). The mixture of 4 mL milk phospholipid dispersion (1 mg/mL) and 1 mL linoleic acid (13%, w/v) were dispersed with sonication for 10 min at 20 °C. Following encapsulation of the oil droplets, the samples were incubated in dark at 50 °C for 2 days. An aliquot (1 mL) was added to 2.5 mL TBA solution (0.375% TBA (w/v), 15% trichloroacetic acid (w/v), and 0.25 M HCl), and was then boiled for 10 min. The thiobarbituric reactive substance, malondialdehyde (MDA), from the oxidation of linoleic acid, became pink. The supernatant after centrifugation (5000g, 25 °C, and 10 min) was then determined spectrophotometrically at 532 nm. Lipid peroxidation inhibition percentage (IR) was calculated by Eq. (6):

$$IR(\%) = [(A_c - A_s)/A_c] \times 100 \quad (6)$$

where A<sub>c</sub> and A<sub>s</sub> were the absorbance of controls and samples,



respectively.

### 2.6.5. Cellular antioxidant activity

The cellular antioxidant activity (CAA) of milk phospholipids was assayed on HT-29 cells as previously reported (Wolfe & Liu, 2007). The cells were seeded in a density of  $5 \times 10^4$ /well in a 96-well microplate. After one day's incubation at 37 °C, the growth medium was removed and the cells were washed twice using PBS. Subsequently, the cells were treated with milk phospholipid dispersion (0.05–1 mg/mL) and DCFH-DA (25  $\mu$ M) for 60 min. Following the removal of growth medium and washing twice with PBS, 600  $\mu$ M 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP, Sigma-Aldrich) was applied to the cells in 100  $\mu$ L of growth medium. The emission at 538 nm was measured with excitation at 485 nm in one hour, using a microplate reader. The CAA value was calculated as Wolfe and Liu (2007).

### 2.7. Statistical method

The averages of all results were obtained from triplicate measurements. Statistical analysis of results was carried out by one-way analysis of variance (ANOVA) with a Tukey test using Minitab 18 software (Minitab Inc., Chicago, USA). Means were considered as significantly difference at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Chemical composition analysis

The total phospholipid content of milk samples was  $12.80 \pm 1.30\%$  (w/w), and the dry-matter purity was  $91.14 \pm 1.56\%$  (w/w) after extraction. The relative protein and triacylglycerol contents were  $4.50 \pm 1.18\%$  and  $4.35 \pm 1.71\%$ , respectively. The purity of phospholipids obtained in this study was higher than that of Fonterra's Phospholac 600 and Phospholac 700 (Li, 2014), and Arla's Lacprodan® PL-75 (Phan, 2015). The increased purity of milk phospholipids observed in this study was due to the repeated defatting by acetone.

### 3.2. Chromatographic analysis of milk phospholipids

The phosphatidylcholine (PC) band was first located by aligning its  $R_f$  with that of standard PC, then the others were determined by their ranking of  $R_f$ . As illustrated in Fig. 1, the bands appeared in an ascending order sphingomyelin (SM,  $R_f = 0.21 \pm 0.00$ ), phosphatidylserine (PS,  $R_f = 0.34 \pm 0.00$ ), PC ( $R_f = 0.62 \pm 0.02$ ), phosphatidylethanolamine (PE,  $R_f = 0.88 \pm 0.01$ ), and phosphatidylinositol (PI,  $R_f = 0.95 \pm 0.04$ ). The comparative areas of each spot were measured with ImageJ Software (National Institutes of Health, Bethesda, MD, USA), resulting in a relative content of phospholipid species (SM  $12.5 \pm 1.8\%$ , PS  $17.7 \pm 0.5\%$ , PC  $36.9 \pm 2.9\%$ , PE  $19.6 \pm 5.3\%$ , PI  $9.5 \pm 1.0\%$ ). The constituent of milk phospholipids by TLC analysis in Fig. 1 was comparable to a recent report using HPLC evaporative light scattering detector (ELSD) assay (Et-Thakafy, Guyomarc'h, & Lopez, 2017). Also, bovine milk phospholipid sub-classes have been analysed with  $P^{31}$  Nuclear Magnetic Resonance (NMR) (Garcia et al., 2012), with similar results except for the composition of PE, which may be due to the discrepancy in both samples and assays.

### 3.3. Milk phospholipid digestion and cellular uptake

#### 3.3.1. FFA from milk phospholipids and triacylglycerols

As illustrated in Fig. 2(a), both milk phospholipids and triacylglycerols were digested following first-order kinetics, but with different reaction velocity and degrees. In the first 20 min, approximately 56% of fatty acids were released from milk polar lipids, much higher than that of neutral lipids. Following 2 h of intestinal digestion, the hydrolysis degrees were  $91.60 \pm 4.04\%$  and  $56.71 \pm 3.51\%$  for phospholipids

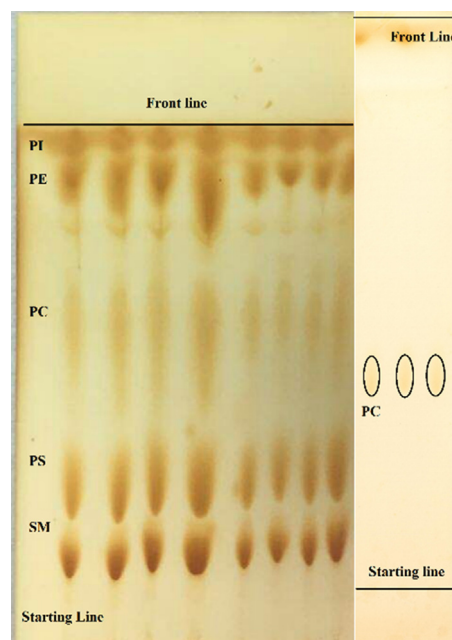


Fig. 1. The chromatogram of milk phospholipids (MPL) and standard phospholipids (PC, right), and the relative position was determined by aligning the  $R_f$  value of each band with that of the reference. Phosphatidylinositol (PI), phosphatidylethanolamine (PE), Phosphatidylcholine (PC), phosphatidylserine (PS), and sphingomyelin (SM) were visualized from top to bottom.

and triacylglycerols, respectively. Their breakdown rate constants were  $0.016 \text{ min}^{-1}$  and  $0.007 \text{ min}^{-1}$ , corresponding to a half-digestion time of 6.35 min and 43.86 min, respectively. The results are comparable to a previous report on lipolysis kinetics by type II lipase with L- $\alpha$ -phosphatidylcholine (Sigma-Aldrich, 1%, w/w) (Mutsokoti et al., 2017).

The lipolysis abided by first-order kinetics ( $R^2 = 0.98$  and  $0.99$  for phospholipids and triacylglycerols, respectively), consistent with reports on hydrolysis of palm, rapeseed, and linseed oil (Ye, Cao, Liu, Cao, & Li, 2018) and carotenoid enriched olive oil in phosphatidylcholine emulsion (Mutsokoti et al., 2017). Milk phospholipids exhibited a higher digestion velocity and hydrolysis degree than triacylglycerols, indicating the polarity influence on the reaction rate constant. The difference in the kinetics might be due to several factors, such as the difference in lipase catalysis and interfacial properties between phospholipids and triacylglycerols.

Dietary phospholipids remain intact in the presence of lingual and gastric lipases (Castro-Gómez et al., 2015). Gastric pH and salts can alter both the size and the structure of particles, which further affect the intestinal digestion. However, gastric digestion contributes to only a minor proportion (1.5–8%) of the total phospholipid hydrolysis (van Hoogevest, 2017) and takes place at pH 1.5–3, which is incompatible with the present titration quantification since both gastric acid and free fatty acids consume titrant. Therefore, only intestinal digestion was surveyed this study, as also commonly performed by recent reports on lipid digestion (Guo, Bellissimo, & Rousseau, 2017; Kaimainen, Marze, Järvenpää, Anton, & Huopalahti, 2015; Saele, Rod, Quinlivan, Li, & Farber, 2018). AACC 02-31.01 method (AACC, 2010) was used to titrimetrically quantify FFA. The titrimetric acidity of small amount of lipophilic solution can be best detected with ethanolic potassium hydroxide in the presence of diethyl ether (Saad et al., 2007), as also adapted in an AOAC alternative (Hur et al., 2009).

In humans, pancreatic phospholipase  $A_2$  (EC 3.1.1.4) can act upon the *sn*-2 position of phospholipids, resulting in lysophospholipids and fatty acids (Venuti et al., 2017). The fatty acid group of lysophospholipids can be further cleaved by lysophospholipase (EC 3.1.1.5)

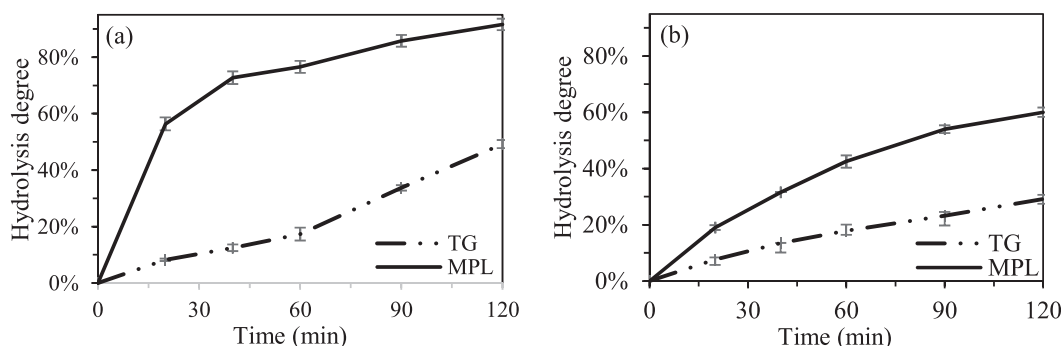


Fig. 2. Milk lipolysis by pancreatic lipase (PL) (a) and fungal lipase Lipopan F (b); MPL: milk phospholipids; TG: triacylglycerols.

(Sugimoto, Hayashi, & Yamashita, 1996). Moreover, the pancreatic lysophospholipase of human being is most likely a non-specific phospholipase, that is, carboxyl ester hydrolase (CEH, EC 3.1.1.1, wide substrate specificity) (Duan & Borgström, 1993). In addition, CEH can also attack intact phospholipids (Aranda et al., 2014), and whereas, triacylglycerol lipase (EC 3.1.1.3) acts on triacylglycerols concomitantly with co-lipase, in the presence of bile salt (Ye et al., 2018). In brief, different enzymes are involved in the hydrolysis of triacylglycerols and phospholipids during intestinal digestion, and thereby their reaction rate constants differ from each other.

In addition of pancreatic lipase, Lipopan F fungal lipase (EC 3.1.1.X, wide substrate specificity, Novozymes A/S, Bagsvaerd, Denmark) was used to break down phospholipids and triacylglycerols in the present study. As illustrated in Fig. 2(b), the lipolysis reaction rate constants under the fungal lipase catalysis were  $0.002 \pm 0.001 \text{ min}^{-1}$  and  $0.007 \pm 0.001 \text{ min}^{-1}$  for triacylglycerols and phospholipids, respectively, corresponding to half digestion time of  $289.80 \pm 21.52 \text{ min}$  ( $R^2=0.96$ ) and  $97.69 \pm 8.88 \text{ min}$  ( $R^2=0.97$ ). Since phospholipid hydrolysis was faster than that of triacylglycerols in both specific lipase and non-specific lipase (Lipopan F), there must be factors other than lipases that contributed the reaction rate difference of the two lipolysis reactions.

Lipolysis occurs by interfacial catalysis, which involves two equilibrium processes. Firstly, the adsorption of the lipase to the lipo-aqueous interface, followed by the subsequent conformation of the acyl-lipase complex intermediate (Aranda et al., 2014). The lipase must penetrate the interface to act, therefore the interfacial properties of substrates (e.g. in the form of micelles, emulsions, liposomes, or vesicles), have a direct impact on lipolysis kinetics (Wong, 1995). In addition to colloidal structure, the lipolysis reaction rate constant is also dependent on lipid composition. For instance, short chain fatty acids hydrolyse faster than long chain fatty acids, and whereas, milk fat breaks down faster than soy and fish oil (Zhang et al., 2015). Despite the presence of short- (ca. 1%) and medium-chain fatty acids (ca. 8%) in bovine milk triacylglycerol that are almost not found in bovine milk phospholipids, predominant fatty acids of both milk phospholipids and triacylglycerols are oleic, palmitic, stearic and myristic acids, accounting for around 80% of the total fatty acids. Further, unsaturation degree appears to be an insignificant factor on *in vitro* lipolysis (Ye et al., 2018), and a higher degree of unsaturated lipid (C18:1 and C18:2) digests more effectively due to elevated cholecystokinin (CCK) response during *in vivo* digestion. The colloidal difference may be the key factor that caused the greater phospholipid digestion. Further, larger surface area and cation-phospholipid association contribute to the quick access of the lipase to the substrate (Akoh & Min, 2017).

In regard to lipolysis mechanism, phospholipids may enhance micellarisation and solubilisation of hydrolysis products in micellar phase (Bezulgues, Morgan, Palomo, Crosset-Perrotin, & Ducret, 2009). Both lipid composition and emulsion properties have impact on the digestibility of lipid (Singh, Ye, & Horne, 2009); the triacylglycerol aggregates formed in small intestine may have been resistant to digestion

(Zhang et al., 2015). Additionally, an *in vivo* study has reported appreciable differences in the release of free fatty acids depending on whether they are in triacylglycerol or phospholipid forms—pancreatic lipase hydrolyses molecules of triacylglycerols towards positions of *sn*-1 and *sn*-3 to yield two molecules of fatty acids and a molecule of 2-monoacylglycerols, and whereas, phospholipids breakdown from positions of *sn*-1 and *sn*-2. Further, interfacial mechanism also play a role in lipolysis, and it appears that bile salts-phospholipids interaction can impede liquid condensed phase and augment liquid expanded phase, which in turn, improves the accessibility of lipase to lipid (Wilde & Chu, 2011).

Previously, bovine milk phospholipids have been refined to boost cognitive performance of infants (Sokol et al., 2015) as claimed by manufactures, which have been evidenced by either by *ex vivo* models (suckling rats pups (Brink & Lönnardal, 2018) and neonatal piglet (Liu et al., 2014) or by *in vivo* models low birth weight infants (Tanaka et al., 2013), infants (Gurnida, Rowan, Idjradinata, Muchtadi, & Sekarwana, 2012), and toddlers (Timby, Domellof, Hernell, Lönnardal, & Domellof, 2014). The intestinal secretion of pancreatic triacylglycerol lipase (PTL, EC 3.1.1.3), pancreatic phospholipase A<sub>2</sub> (EC 3.1.1.4), and bile salt are less in newborns than those of adults. Instead PTL-related protein 2 and bile salt-stimulated lipase (BSSL, also present in breast milk) are the key lipases responsible for digesting both phospholipids and triacylglycerols in babies' milk in conjunction with gastric digestion (Lindquist & Hernell, 2010).

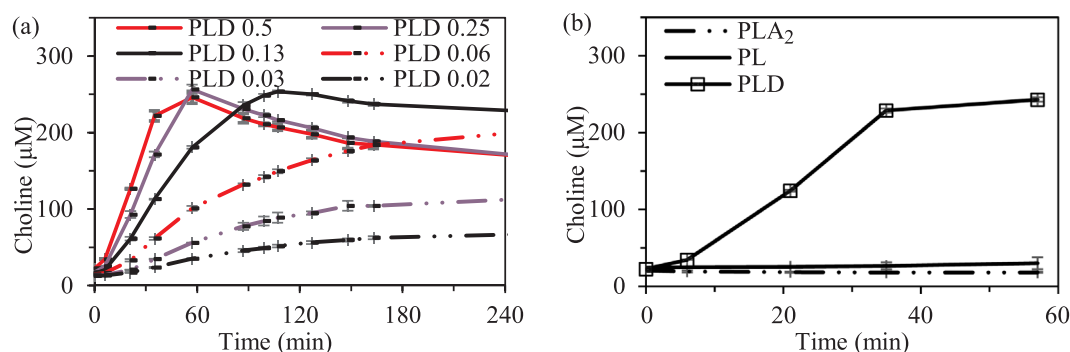
### 3.3.2. Cleavage of phospholipids to release choline

Choline phospholipid breakdown by phospholipases D and A<sub>2</sub>, and pancreatic lipases is illustrated in Fig. 3(b) at a constant concentration of substrates and enzymes. Phospholipase D (PLD, EC 3.1.4.4) cleaves the choline polar heads of the phosphoric di-ester bonds at the *sn*-3 positions, and can also catalyse transphosphatidylations reactions (Vines & Bill, 2015). Phospholipase A<sub>2</sub> present in pancreatic juice specifically attacks the *sn*-2 acyl position (Venuti et al., 2017). PLD, an intracellular enzyme absent in pancreatic juice, may be present in dietary ingredients, e.g. cabbage and carrots. Hence phospholipase A<sub>2</sub> and pancreatic lipase had no cleavage effects on choline groups of phospholipids in the present study.

Aside from choline-cleaving phospholipase D, sphingomyelinase (alk-SMase, EC 3.1.4.12) acts on the phosphoric di-ester bond of sphingomyelin, generating ceramide and phosphocholine (Nilsson & Duan, 2019). Ceramide will be further disintegrated by mucosal ceramidase (N-CDase EC 3.5.1.23) (Mao & Obeid, 2008).

### 3.3.3. Choline phospholipid digestion with PLD

Choline released from milk phospholipids exhibited first-order reaction kinetics under the catalysis of PLD, as illustrated in Fig. 3(a). The reaction rate was proportional to the first power of substrate concentration. As the enzyme concentration changed, the reaction rate constants also changed in a range of  $0.031\text{--}0.001 \text{ min}^{-1}$ , leading to half-digestion time ranging from 22 to 495 min, as recorded in Table 1.



**Fig. 3.** Choline cleavage of milk phospholipids (MPL, 1 mg/mL): (a) by various concentrations of phospholipase D (PLD, mg/mL); (b) by PLD, phospholipase A<sub>2</sub> (PLA<sub>2</sub>), and pancreatic lipase (PL, 1 mg/mL).

**Table 1**

Choline cleavage reaction rate constants and half-digestion time of phospholipase catalysis on milk phospholipids (MPL, 1 mg/mL).

PLD concentration (mg/mL)	K ( $\times 10^{-2} \text{ min}^{-1}$ )	R <sup>2</sup>	T <sub>1/2</sub> (min)
0.50	2.7 $\pm$ 0.1	0.96	25.6 $\pm$ 0.58
0.25	3.2 $\pm$ 0.0	0.94	21.5 $\pm$ 0.00
0.13	1.6 $\pm$ 0.0	0.97	43.2 $\pm$ 1.13
0.06	0.6 $\pm$ 0.0	0.96	120.9 $\pm$ 1.21
0.03	0.3 $\pm$ 0.0	0.96	266.5 $\pm$ 0.00
0.02	0.13 $\pm$ 0.1	0.96	533.1 $\pm$ 0.00

Notes: k: reaction rate constant; T<sub>1/2</sub>: half-digestion time (min); PLD: phospholipase D.

Also, similar first-order reaction kinetics have been documented in reports on lipolysis of carotenoid-enriched lipids (Verkempinck et al., 2018) and on the lipolysis of nano-emulsion (Salvia-Trujillo et al., 2019). PLD plays critical roles in a wide varieties of physiological pathways, for instance, membrane trafficking, cytoskeletal reorganisation, regulation of cell proliferation and transformation, and a biomarker of neurodegenerative diseases (i.e. Parkinson's and Alzheimer's) (Foster & Xu, 2003). Two isoforms PLD<sub>1</sub> (120 kDa) and PLD<sub>2</sub> (106 kDa) have been characterized, and the latter can be derived from plants (e.g. cabbage and carrots), animals (e.g. brown spider and venom), and bacteria (e.g. *Ochrobactrum anthropi*) (Vines & Bill, 2015).

### 3.4. Cellular uptake of phospholipids on HT-29 cells

As illustrated in Fig. 4(f), the cellular uptake of milk phospholipids on HT-29 cells was 6.1  $\pm$  0.7%, in line with previous reports on direct diffusion across cell lines. For instance, lipolysates (fatty acids and lysophospholipids) are absorbed across epithelial cells, with an absorption rate of more than 90%, and approximately 20% can actively diffuse the mucosa brush border (Castro-Gómez et al., 2015). Also, liposomes are biocompatible, and are able to penetrate into epithelial cells (Arranz & Corredig, 2017). Most phospholipids are not absorbed intact, and must be digested to permeate the epithelial cells (Nilsson & Duan, 2019). Additionally, the uptake of fatty acids (hydrolysates of triacylglycerols and phospholipids) is mediated by very low density lipoproteins (Tamime, 2009). Cell membranes are permeable for small molecules, and lipid micelles are too large to cross the cell membrane actively by diffusion (Zhao et al., 2011).

### 3.5. Milk phospholipid antioxidant activity

As illustrated by the DPPH assay (a), hydroxyl radical assay (b) and ABTS assay (c) in Fig. 4, the antioxidant activity of milk phospholipids was concentration-dependent, in line with previous reports on oil oxidation inhibition by phospholipids. For instance, phospholipids of egg yolk PC and ox brain PE protected *n*-3 polyunsaturated fatty acids

(PUFAs) of salmon oil from peroxidation during storage (Nwosu, Boyd, & Sheldon, 1997). Further, 0.5% phospholipid supplementation was found to remarkably alleviate the concentration of volatile organic compounds generated from both salmon and menhaden oils (Body, Nwosu, Young, & MacMillan, 1998). The primary substrates of free radical reactions include the PUFA moieties (King, Boyd, & Sheldon, 1992), amine groups (of PC, SM, PE, or PS) and hydroxyl groups (of phosphatidylglycerol (PG) or PI) in the *sn*-3 side chain of phospholipids (Lu, Nielsen, Timm-Heinrich, & Jacobsen, 2011). Also, the moieties of hydroxyl and nitrogen-containing amine groups collectively act as catalysts of hydroperoxide decomposition (Saito & Ishihara, 1997), and therefore, PC, PE, and SM exhibit more antioxidant activity than PS, PG and PI. Whereas, phosphatidic acid (PA) demonstrated no antioxidant activity (King et al., 1992). TBARS results 4(d) further evidenced that the antioxidant activity of milk phospholipids can be used to delay the oxidation of PUFAs.

The CAA assay was developed recently to determine the attenuation effects of antioxidants on the cellular oxidation. As illustrated in Fig. 4(e), the CAA value of milk phospholipids was very low, indicating a poor bioavailability of the antioxidants. The intracellular DCFA was oxidized to fluorescent DCF, which can be reduced with intracellular antioxidants (Meng et al., 2017). Further tests using *in vivo* or *ex vivo* models (rat/mice models) may be worthwhile for assessment of antioxidant activity of milk phospholipids.

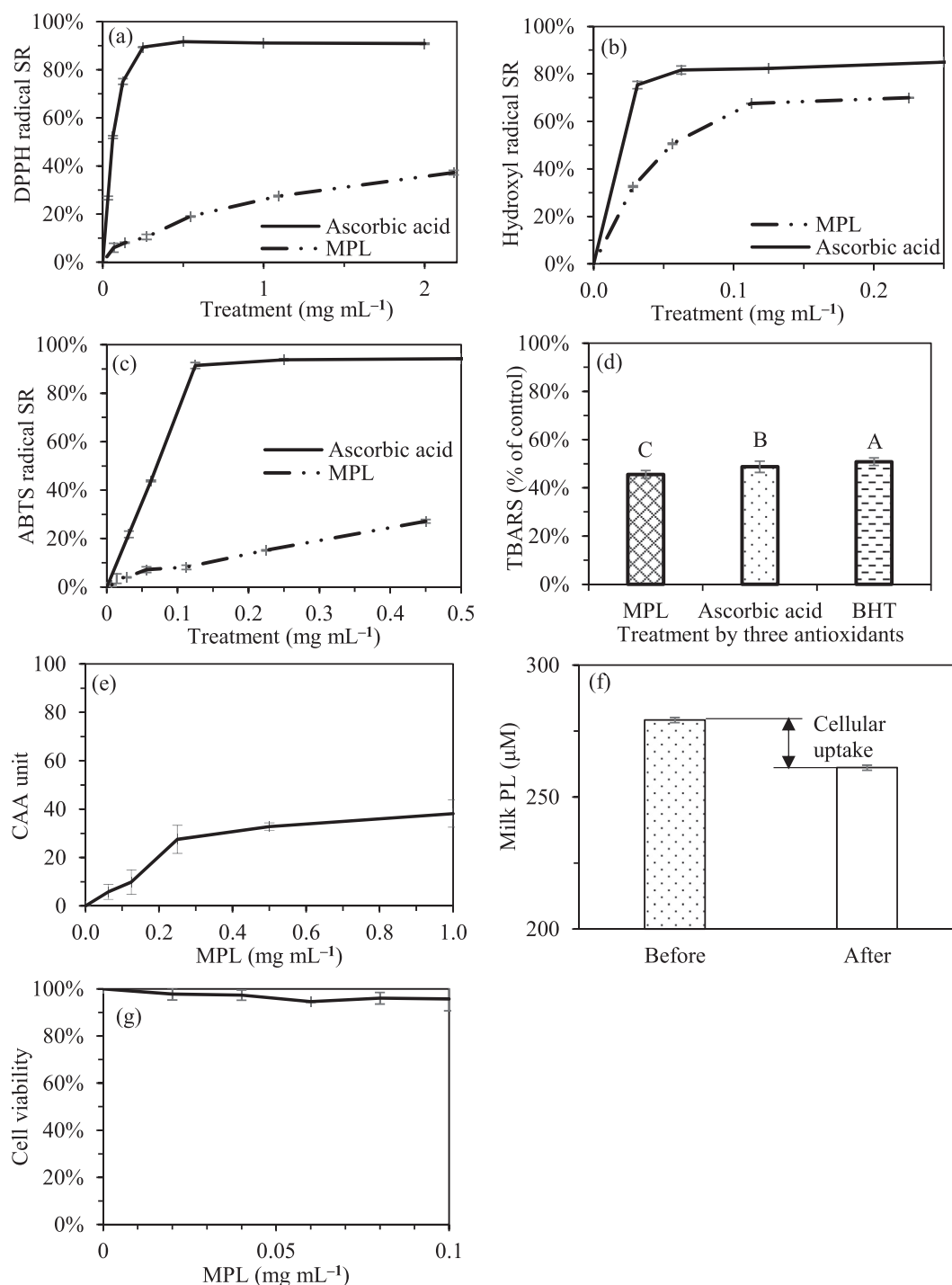
Milk phospholipids are increasingly regarded as important nutritional ingredients for infant milk formulas. In this study, they showed a degree of *in vitro* antioxidant activity which, in addition to its nutritional benefits, may also be valuable for milk powder preservation during shelf life.

### 3.6. Anti-proliferation activity

Fig. 4(g) illustrates that milk phospholipids have no statistically significant effect on HT-29 cell viability. This demonstrates their biocompatibility to HT-29 cells, and is consistent with previous reports on soy lecithin and bovine brain PS. For instance, oral administration of PS exhibited no adverse effects on models of both *in vivo* (Jorissen, Brouns, Van Boxtel, & Riedel, 2002) and *ex vivo* (Heywood, Cozens, & Richold, 1987). Also, long-term feeding studies of soy lecithin yielded no treatment-related histologic changes in mice (Myers et al., 1993) and rats (Brantom, Gaunt, Hardy, Grasso, & Gangolli, 1973).

## 4. Conclusion

In conclusion, milk phospholipid lipolysis abided by first-order reaction kinetics, which were significantly higher ( $p < 0.05$ ) than those of triacylglycerols by pancreatic lipase and fungal lipase. Significant antioxidant activity was evidenced by *in vitro* assays, but their cellular antioxidant activity was very limited. Additionally, their choline groups were not cleaved by pancreatic lipase and they were not absorbed intact



**Fig. 4.** Milk phospholipid (MPL) antioxidant activity by DPPH radical scavenging (a), hydroxyl radical scavenging (b), ABTS radical scavenging (c), TBARS formation inhibition (d), Cellular antioxidant activity (CAA) assays (e), MPL concentration before and after cellular uptake (f), and relative cell viability to untreated HT-29 cells by MTT assay (g).

during intestinal digestion. This study elaborates the polarity effects on lipid digestibility and provides useful knowledge on the design of milk phospholipid-fortified functional foods including infant formulas.

#### Conflict of interest

The authors have declared no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2020.103865>.



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